Government of Canada / Gouvernement du Canada
Canadian Forestry Service / Service canadien des forêts

Monoclonal antibodies and immunochemical techniques : applications in forestry research

Leslie Ann Mitchell

Information Report BC-X-258
Pacific Forest Research Centre

85-5471/

Monoclonal antibodies and immunochemical techniques: applications in forestry research

Leslie Ann Mitchell[1]

Canadian Forestry Service
Pacific Forest Research Centre

BC-X-258

1985

[1] Leslie Ann Mitchell is a Natural Sciences and Engineering Research Council Visiting Fellow at the Pacific Forest Research Centre

Canadian Forestry Service
Pacific Forest Research Centre
506 West Burnside Road
Victoria, B.C.
V8Z 1M5

© Minister of Supply & Services Canada, 1985
ISSN 0705-3274
ISBN 0-662-13593-8
Cat. No. Fo46-17/258E

Abstract

Immunologic techniques employing polyclonal antisera and monoclonal antibodies as sensitive, highly specific probes for molecules in complex biological systems are currently in use in many research laboratories. For those forestry researchers unfamiliar with these techniques, this report describes polyclonal antisera and monoclonal antibodies and the principles behind their use in sensitive techniques such as radioimmunometric assay (RIA), enzyme-linked immunosorbent assay (ELISA), immunofluorescence, immunochemical localization of structures in light or electron microscopy, and immunoaffinity chromatography. The use of antisera and monoclonal antibodies in conjunction with other high-resolution techniques (such as single- and two-dimensional polyacrylamide gel electrophoresis) in immunoprecipitation and immunoblotting (Western blotting) techniques is also described. The contribution of monoclonal antibodies and antisera to gene identification and localization in genetic engineering is discussed. Examples of current and potential applications of these immunologic techniques in agricultural and forestry research are given.

Résumé

Des techniques immunologiques utilisant des immuns-sérums polyclonaux et des anticorps monoclonaux comme outils sensibles et extrêmement spécifiques d'analyse des molécules dans des systèmes biologiques complexes sont employées dans de nombreux laboratoires de recherche. Nous décrivons a l'intention des chercheurs dans le domaine forestier qui ne seraient pas familiers avec ces techniques ce que sont ces immuns-sérums et ces anticorps et les principes de leur utilisation dans des techniques sensibles comme les dosages radio-immunologiques (RIA), les tests immuno-enzymatiques (ELISA), l'immunofluorescence, la localisation immunochimique de structures sous microscope optique ou électronique et l'immunochromatographie d'affinité. Nous traitons également des techniques d'immunoprécipitation et des techniques immunologiques avec transfert sur papier ("Western blotting techniques") qui font appel à d'autres techniques à haute résolution (comme l'électrophorese unidimensionnelle et bidimensionnelle en gel de polyacrylamide). Nous examinons l'apport des anticorps monoclonaux et des immuns-sérums à l'identification et à la localisation des gènes en génie génétique. Nous étudions des exemples d'applications actuelles et potentielles de ces techniques dans la recherche intéressant les domaines agricole et forestier.

Table of Contents

	Page
Introduction	6
The immune response	6
Antigens	6
Antibodies	7
Antisera	9
Monoclonal antibodies	10
Monoclonal antibodies vs. antisera	10
Procedure for producing monoclonal antibodies	10
Advantages of monoclonal antibodies	13
Immunochemical techniques and their applications	13
Radioimmunometric assay (RIA)	14
Enzyme-linked immunosorbent assay (ELISA)	14
Other immunoassays	14
Immunofluorescence and immunohistochemistry	18
Immunoaffinity chromatography	19
Immunoblotting techniques	19
Immunoprecipitation	20
Two-dimensional polyacrylamide gel electrophoresis	21
Genetic engineering	23
Conclusion	23
References	24

Figures

		Page
1	Antigens	6
2	Antibody structure	8
3	Clonal selection: An antibody response to a multivalent antigen	8
4	Clonal expansion	9
5	Preparing an antiserum	9
6	Monoclonal antibodies vs. antisera	11
7	Procedure for producing monoclonal antibodies	12
8	Radioimmunometric assays (RIAs)	14
9	Enzyme-linked immunosorbent assays (ELISAs)	15
10	Sandwich assays	16
11	Competitive inhibition assays	16
12	Immunoaffinity chromatography	18
13	Immunoblotting	19
14	Immunoprecipitation	20
15	Two-dimensional polyacrylamide gel electrophoresis of mouse serum proteins	21
16	Monoclonal antibodies and genetic engineering	22

Tables

		Page
1	Some properties of human antibody classes	7

Introduction

Forest biology researchers often require sensitive, rapid and inexpensive methods for locating, identifying and measuring both microorganisms and biochemicals in plant and insect tissues, soils or the environment. For example, accurate detection methods are needed for pathogens such as bacteria and fungi in seeds, seedlings and mature tree tissues so that early and appropriate control measures may be taken and also for seed certification. Sensitive methods are also required for monitoring levels of pathogens such as fungi or levels of biologic control agents such as viruses in insect tissues for the evaluation and management of insect-borne disease or biological control programs. Forestry researchers are also seeking high-resolution techniques to facilitate studies of species relationships (for instance, among insect pests, seeds of different origin, pollens and races of fungal pathogens differing in virulence or host range) or to quantitate growth regulators (e.g., cytokinins influencing cone production) or other biochemical changes such as the synthesis of phytoalexins in host tissues in response to fungal, bacterial or other pathogens Forest and agriculture biologists are also developing techniques which permit the identification of proteins (or other biological substances) associated with disease resistance to facilitate the classification of superior genetic stock and ultimately to identify and isolate resistance genes for use in genetic engineering.

Sensitive, high-resolution techniques originally developed for the study of human and animal systems are being adapted for use in agriculture and forestry research. Immunologic techniques employing antisera or monoclonal antibodies, used alone or in conjunction with other high-resolution systems (such as polyacrylamide gel electrophoresis) constitute one group of these newly adapted technologies. Although they are not without some problems and limitations, these techniques are very powerful and extremely sensitive.

This information report, which is directed at forestry researchers who are unfamiliar with immunologic techniques, describes the biochemical nature and derivation of antisera and monoclonal antibodies and their use as sensitive and specific probes in a variety of procedures.

The immune response

Immunology made its first impact on humanity when it was observed that vaccination with live or heat-killed microorganisms would protect people from the diseases caused by these pathogens. The word "immunology" derives from the Latin "immunitas" meaning "freedom from public service" which later came to be associated with freedom from disease. Although immunology originally dealt with the body's response to disease organisms, it soon became evident that all vertebrates (and some invertebrates) are capable of recognizing foreign substances and of making specific responses against them.

Antigens

Foreign substances that induce immune re sponses are called antigens. Almost any substance may be antigenic (immunogenic) if the animal's immune system perceives it as foreign. Antigens may be proteins, lipids, carbohydrates or even small molecules. For practical purposes, an antigen may be considered as any substance that a researcher might wish to detect using immunological techniques (e.g., a virus, fungus, enzyme, growth regulator or insect component).

Most antigens are multivalent, i.e., they contain several regions called antigenic sites (Fig. 1). Each antigenic site on the antigen molecule is capable of stimulating a different aspect of the immune system.

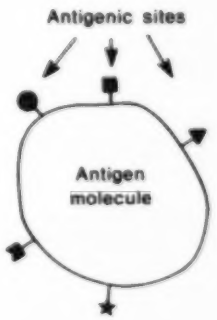


Fig. 1. Antigens. An antigen is a foreign molecule which, upon entering the body, induces an immune response. Most antigens are multivalent, i.e., they have several antigenic subregions called antigenic sites.

Antibodies

White blood cells called lymphocytes respond to foreign substances entering our bodies. These cells are found in lymphoid organs such as the spleen, thymus gland, lymph nodes, tonsils and appendix as well as in the blood.

Antigens cause some lymphocytes to proliferate and change into cells which synthesize and secrete substances called antibodies (or immunoglobulins, abbreviated "Ig"). The antibodies bind specifically to the foreign molecule and either neutralize its activity or clear it from the body. Antibodies are fairly large, multichain carbohydrate-containing proteins ranging in molecular weight from 150 000 to 900 000 Daltons. There are several different classes of antibodies. Some physical and biological properties of human antibodies are listed in Table 1. The immunoglobulin classes differ in molecular weight, carbohydrate content and serum half-life.

Antibodies are found in various places within the body: in the blood, lymphoid organs, gut and respiratory tract linings. Different classes of antibodies function somewhat differently in the clearance of foreign substances from the body. Also, more than one class of antibody can be generated in an immune response. However, all antibody classes have one thing in common: their absolute specificity for the antigenic substance which elicited their formation.

Figure 2 is a schematic representation of an antibody molecule. Antibodies of all classes are made up of similar unit structures. For example IgG antibodies resemble the structure shown in Figure 2, while IgM antibodies contain five such units linked together as a pentamer. An antibody molecule is composed of two light and two heavy polypeptide chains held together by disulfide bonds. The sequence of amino acids in the amino-terminals (N_T) of the heavy and light chains are unique for a given species of antibody while the remaining amino acids are constant for a given heavy chain class or light chain type. The unique sequences of amino acids form the antigen binding site of the antibody. There are two identical binding sites per antibody monomer as represented in Figure 2. They are folded in such a way that they are complementary to a particular antigenic site on the antigen to which they bind. Thus, the size, shape and amino acid lining of the antigen binding site are different for every antibody. This provides antibody molecules with their unique specificites. Therefore, different molecular antibody species are able to recognize unique regions on foreign substances which are not distinguishable by other means. For exam-

Table 1. Some properties of human antibody classes

Antibody class	Molecular weight (Daltons)	Carbohydrate content (%)	Concentration in serum (mg/ml)	Half-life (days)
IgG_1	146 000	2–3	9	12±5
IgG_2	146 000	2–3	3	20±2
IgG_3	165 000	2–3	1	7±1
IgG_4	146 000	2–3	0.5	21±2.5
IgM	970 000	9–12	1.2	5
IgA	160 000	7–11	2.0	6
IgD	170 000	10–12	0.06	3
IgE	188 000–196 000	12	0.0002	2.5–4

Adapted from Nisonoff (1982)

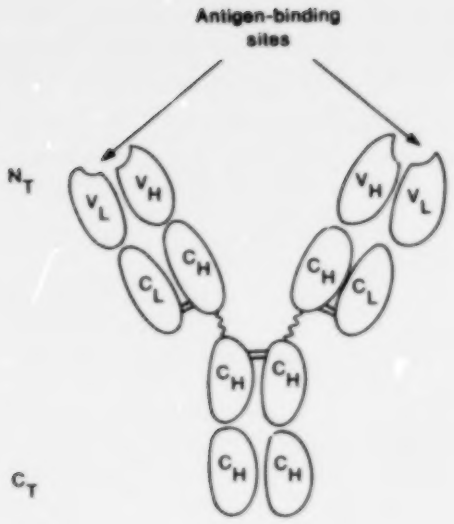


Fig. 2. Antibody structure. The immunoglobulin G (IgG) molecule represented here is the prototype of antibody structure. It consists of two light chains (L) and two heavy chains (H) covalently-linked. The amino-terminal (N_T) regions of both light and heavy chains are highly variable (V) in amino acid sequences, while the carboxy-terminal (C_T) ends of the chains are relatively constant (C) for a given class of antibody molecule.

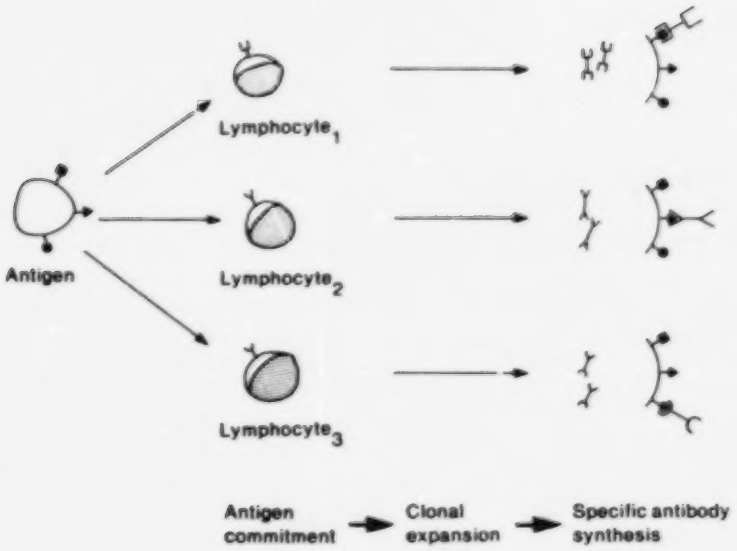


Fig. 3. Clonal selection: an antibody response to a multivalent antigen. When a multivalent antigen is injected into an animal, each antigenic site is recognized by a different lymphocyte which bears on its surface (as an antigen receptor) antibody molecules which fit onto that particular antigenic site. When the surface antibody (receptor) on the lymphocyte binds to the antigenic site, the lymphocyte is triggered into cell division (clonal expansion) and antibody synthesis.

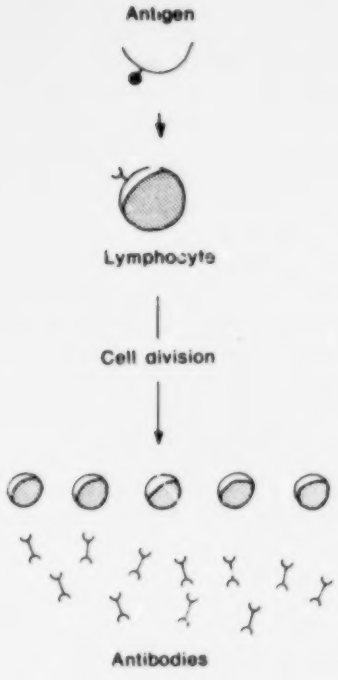


Fig. 4. Clonal expansion. A lymphocyte upon interacting with an antigenic site undergoes cell division giving rise to identical daughter lymphocytes. This lymphocyte clone synthesizes antibodies which are identical to that expressed on the surface of the parent lymphocyte.

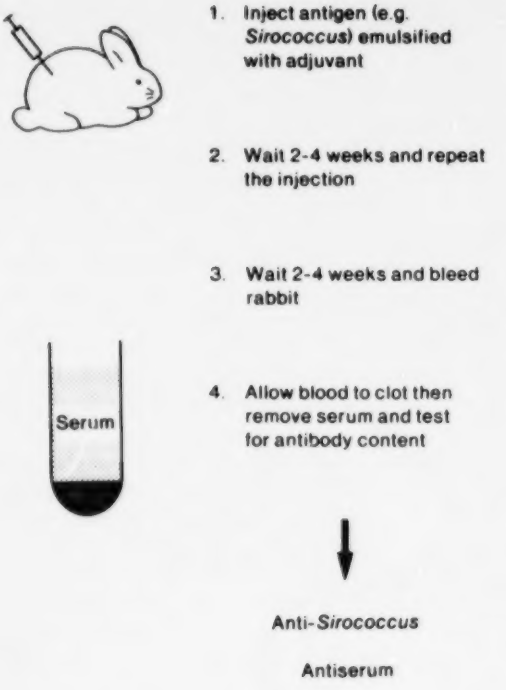

1. Inject antigen (e.g. *Sirococcus*) emulsified with adjuvant
2. Wait 2-4 weeks and repeat the injection
3. Wait 2-4 weeks and bleed rabbit
Serum
4. Allow blood to clot then remove serum and test for antibody content
Anti-*Sirococcus*
Antiserum

Fig. 5. Preparing an antiserum. An antiserum is prepared by injecting an animal (mice, rats, rabbits, goats, sheep are often used) with antigen (e.g. the fungus, *Sirococcus strobilinus*) mixed with adjuvant, an agent which enhances the immune response. The animal's lymphocytes synthesize and secrete antibodies which accumulate in the blood.

ple, a single antibody species may be able to distinguish between closely related viral subtypes thus allowing variants to be identified.

Antisera

Most foreign molecules entering our bodies have more than one antigenic site on their surfaces. Thus, the immune system responds to several different parts of the antigenic molecule. As a result, individual lymphocytes respond differently, each lymphocyte producing antibody of a unique specificity for a single antigenic site. This phenomenon is illustrated in Figure 3. Each lymphocyte triggered by the antigen proliferates (clonal expansion) and its progeny differentiate into cells which synthesize and secrete antibody of the same specificity as the parent lymphocyte (Fig. 4). Therefore, when a foreign molecule enters our bodies the antibody response is heterogeneous (polyclonal) as several different lymphocyte clones will be triggered by different antigenic sites on the same foreign molecule. These antibodies will usually appear in the blood and can be isolated in serum after the red and white cells have been removed. Such a complex mixture of antibodies, which recognizes all parts of the antigen, is called an antiserum.

In the laboratory, antisera are prepared by injecting animals (usually rabbits) with antigen such as the fungus *Sirococcus* (Fig. 5). In order to "tune-up" the animal's immune system the anti-

gen is usually emulsified with an adjuvant (a substance which nonspecifically enhances the immune response). The emulsion also slows down the release of antigen thereby prolonging exposure and increasing the numbers of lymphocytes responding. Usually several injections of antigen are given at two- to four-week intervals. The rabbit is then bled and the serum fraction, which in the example shown in Figure 5 will contain anti-*Sirococcus* antibodies, is collected. In this way, an antiserum can be raised to almost any substance as long as the rabbit's immune system perceives it as foreign. Antisera can then be used in a variety of immunologic tests to identify antigens in complex mixtures or tissues.

One problem with using antisera is that they may not always distinguish between similar antigens (for example, coat proteins obtained from closely related viruses). Because they are mixtures of antibodies directed to all antigenic sites on the antigen, antisera will react with not only unique regions on similar molecules but also common, shared regions. To distinguish between related molecules it is necessary to isolate those antibodies which recognize unique molecular regions.

Monoclonal antibodies

Monoclonal antibodies vs. antisera

A technique devised by Cesar Milstein and Georges Köhler in the mid 1970s (Köhler and Milstein 1975; Köhler *et al.* 1978) has enabled the isolation and immortalization of lymphocyte clones each of which produces antibody of a unique specificity. The antibody molecules produced by each lymphocyte clone bind to a single antigenic site on the antigen used to elicit their formation. Such antibodies are called monoclonal antibodies. In effect, an antiserum could be considered to be a collection of monoclonal antibodies (Fig. 6).

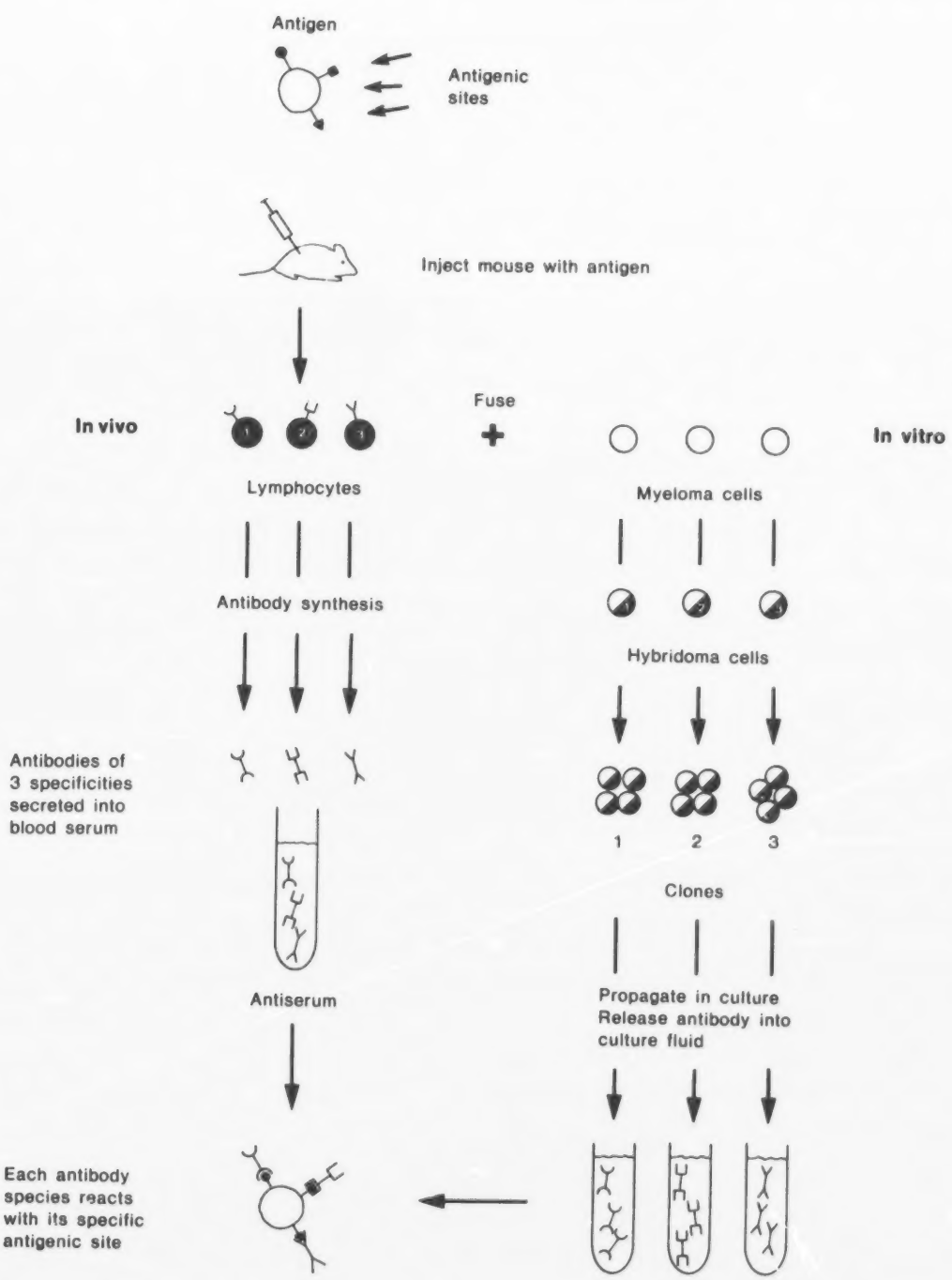
To obtain monoclonal antibodies lymphocytes must be separated from one another after they have been triggered by antigen. It is possible to physically isolate single cells by diluting out lymphocytes in culture medium in microtiter plates so that each well of the plate will contain a single cell (limiting dilution). However, the major drawback is that normal lymphocytes will live for only a few days in a laboratory culture. In contrast, cancerous white blood cells can grow and divide indefinitely in culture. In the technique of Milstein and Köhler, antigen-reactive normal lymphocytes are fused to cancerous white blood cells (myeloma cells) to form a hybrid cell called a hybridoma. The hybridoma acquires the antibody-producing capacity and specificity of the normal lymphocyte and the ability to grow indefinitely in culture from the myeloma partner. When the hybridomas are isolated from each other (by diluting out in culture medium or soft agarose) they proliferate into clones each of which produces monoclonal antibody.

Procedure for producing monoclonal antibodies

The standard procedure for deriving monoclonal antibodies is illustrated in Figure 7. The first step is to immunize a mouse with the desired antigen. Three days after the last injection of antigen, immune lymphocytes are removed from the spleen and mixed with myeloma cells which have been propogated in culture flasks. Polyethylene glycol is added to the cell mixture. This causes the cell surfaces to become sticky and promotes cell fusion. The cells are then dispensed into multiwell culture plates containing a special culture medium called HAT (an abbreviation for hypoxanthine, aminopterin and thymidine). This medium kills all unfused cells and allows only hybridoma cells to survive. After several days culture, the medium from each well is tested for specific antibody which is secreted into the culture medium by the hybridoma cells. At this point more than one type of hybridoma cell (and

→

Fig. 6. Monoclonal antibodies vs. antisera. An antiserum is a mixture consisting of many different species of antibodies (each directed to a different antigenic site on the antigen molecule) because many different lymphocyte clones are triggered by the multivalent antigen. Fusion of antigen-reactive lymphocytes to myeloma cells produces hybrids (hybridomas) which secrete antibody and are able to live indefinitely in culture. Single hybridoma cells may be propagated as clones which synthesize and secrete monoclonal antibody.


Antigen
Antigenic sites
Inject mouse with antigen
In vivo
Fuse
In vitro
Lymphocytes
Myeloma cells
Antibody synthesis
Hybridoma cells
Antibodies of 3 specificities secreted into blood serum
1
2
3
Clones
Antiserum
Propagate in culture
Release antibody into culture fluid
Each antibody species reacts with its specific antigenic site
Antigen
Monoclonal antibodies

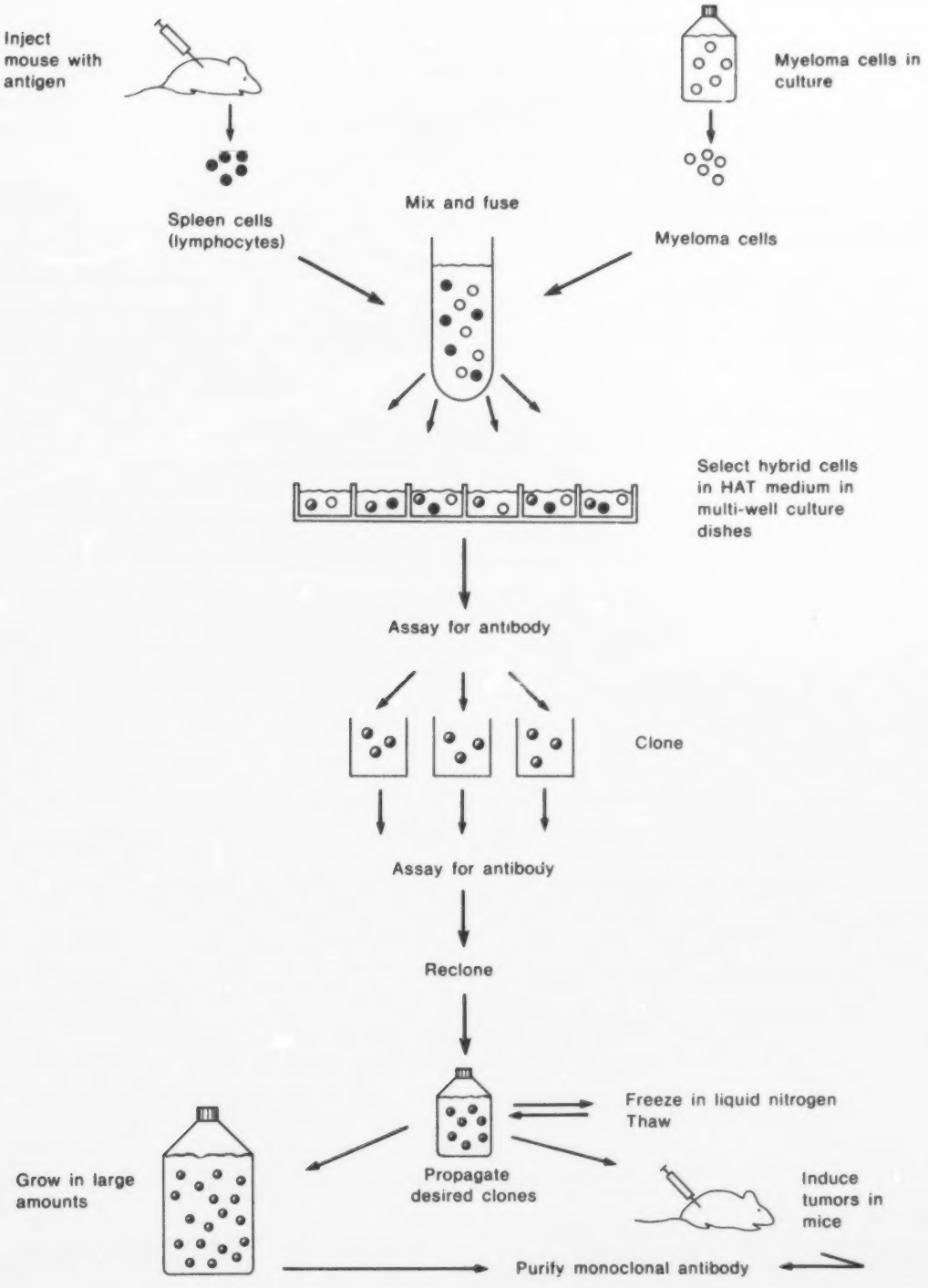


Fig. 7. Procedure for obtaining monoclonal antibodies. See the text for details.

therefore, more than one species of antibody) may be in each well. Cells from each positive well are then cloned by limiting dilution in culture medium. The cells are allowed to proliferate and the clones are retested for antibody production. At this point it is highly probable that each positive clone is derived from a single hybridoma cell. However, to be sure, the cloning process is repeated once more and the wells are retested for antibody production. At this point the monoclonal antibodies from each clone should be characterized biochemically to make sure they are truly different species. After the second cloning, stable clones of interest are grown in culture flasks. Portions are frozen in liquid nitrogen for later use.

Hybridoma cells secrete monoclonal antibody in large quantities into the culture medium. This antibody can be separated from the culture fluid with relative ease. Alternatively, hybridoma cells may be injected into the peritoneal cavities of mice. These cells grow into tumors secreting monoclonal antibody which accumulates as ascites fluid. The ascites may be collected and used directly as a source of monoclonal antibody once the cells have been removed.

Advantages of monoclonal antibodies

The advantages of using monoclonal antibodies over conventional antisera are numerous. Relatively small amounts of antigen are required to immunize a mouse which becomes the donor of the immune lymphocytes and it is not essential that the antigen be pure. These are attractive features when the antigen is in short supply or difficult to purify. Once the hybridoma is established, antigen is no longer required. Therefore, with hybridomas it is possible to produce large amounts of antibody from very small amounts of antigen. In contrast, the production of antisera requires that animals be repetitively immunized with pure antigen.

Frozen hybridoma cells may be thawed and restarted in culture anytime. Monoclonal antibodies can therefore be produced in large quantities whenever needed. In contrast, raising antisera is considerably more expensive and it is necessary to re-immunize the animals at various intervals to maintain a constant supply.

Monoclonal antibodies are singular molecular species and thus are chemically well-defined, pure reagents. In contrast, antisera may show considerable variability. Also, an antiserum raised to one antigen may be partially reactive (crossreactive) with other biochemically related antigens and, as a result, may have to be absorbed by incubation with several crossreactive antigens to render the antiserum monospecific. Thus, the consistency and availability of monoclonal antibodies permits standardized assays to be established.

The greatest advantage of a monoclonal antibody is its specificity. Monoclonal antibodies may be used to identify even minor components in complex mixtures. They are capable of distinguishing between proteins differing in a single amino acid in the antigenic site recognized by the antibody. Such minor differences would probably go undetected with antisera, although the broader specificity of an antiserum may be more desirable for some purposes.

Immunochemical techniques and their applications

Once an antiserum or a monoclonal antibody has been obtained it may be used in a variety of immunologic procedures to detect, isolate or purify antigen in complex mixtures. Two of the most widely used immunologic methods for detecting specific components in complex mixtures are radioimmunometric assay (RIA) and enzyme-linked immunosorbent assay (ELISA). These assays are also used to measure specific antibodies. Immunofluorescence and immunohistochemistry are also used to detect antigen in microscopic procedures, while immunoprecipitation and immunoblotting techniques are used in conjunction with gel electrophoresis to locate and study antigenic substances in biological systems. Antibodies may be used as vehicles for purifying antigen from associated components by immunoaffinity chromatography and also as a means of gene localization in genetic engineering procedures. The principles behind several of these immunologic procedures are detailed below, followed by examples of their applications in plant research.

Radioimmunometric assays (RIA)

Radioimmunometric assays are often used to screen culture fluids for monoclonal antibodies and to test antisera (Fig. 8). These assays take advantage of the property of proteins and other biologic substances to passively adsorb to plastics. The procedure is as follows. Antigen is allowed to adsorb to the wells of polyvinyl chloride microtiter plates. The solutions to be tested for antibody are then added to the antigen-coated wells. Specific antibodies in these solutions bind to the antigen that has been coated onto the plastic wells. The plastic wells are then washed. Specific antibodies remain attached while nonspecific antibodies are washed away. Radioactively-labelled second antibody is then added to detect the antigen-bound first antibody, (i.e.,if the first antibody system was from a mouse, the second antibody would be goat-anti-mouse immunoglobulin prepared by injecting a goat with normal mouse immunoglobulin as antibodies, themselves, may also be antigenic). This second antibody binds to the mouse antibodies and remains bound when the wells are washed. Bound radioactivity is directly proportional to the amount of antigen-specific antibody in the first antibody layer.

Coat wells with antigen (O)
Add solution containing antibodies (ﾒﾝｴｷ) i.e., tissue culture fluids or antiserum
Incubate 1-2 h
Remove fluids and wash wells. Only specific antibodies remain attached.
Add radioactively-labelled second antibody (*)
Incubate
Wash wells and cut
Count wells for bound radioactivity.

Fig. 8. Solid phase radioimmunometric assay. Microtiter plate wells are coated with antigen then overlaid with the solution to be tested for antibodies. Specific antibodies bound to antigen-coated wells are detected with radioactively-labelled second antibody.

Enzyme-linked immunosorbent assays (ELISA)

These assays (Fig. 9) operate on a similar principle to the radioimmunometric assay. Antigen is allowed to passively adsorb to wells of polystyrene microtiter plates. Fluids to be tested for antibody are then added to the antigen-coated wells and incubated for a few hours. The wells are then washed. Again, only specific antibodies remain attached to the antigen while nonspecific antibodies are washed away. Second antibody which has been tagged with an enzyme (such as alkaline phosphatase or horseradish peroxidase) is then added to the wells. The second antibody will bind to antibodies attached to the antigen. Following incubation with the second antibody the microtiter plates are washed and enzyme substrate is added to the wells. Those wells containing bound enzyme-labelled second antibody will be able to convert the substrate into a colored reaction product which can be visually observed or measured spectrophometrically. Fluorogenic substrates may also be used to increase the sensitivity of the assay. Thus, the colored or fluorescent reaction product is quantitatively related to the amount of specific antibody present in the first layer.

Other immunoassays

Either RIA or ELISA may be manipulated to measure an antigen (for example, a virus) in complex biological mixtures. Two frequently used variations are sandwich assays and competitive inhibition assays.

(i) Sandwich assays: In these assays (Fig. 10) antibody specific for the antigen of interest (for example, a monoclonal antibody to a plant virus) is coated onto the plastic microtiter plate wells. Next, the solution to be tested (for example, an extract obtained from a leaf, seed or insect suspected of harboring the virus) is added to the antibody coated wells. The virus, if present in this complex mixture, will be pulled out by the antibody adhering to the well even though the tissue extract contains many other substances. When the plates are washed the virus will remain attached to the antibody. The adherent virus can then be detected by adding radioactively-labelled or enzyme-labelled monoclonal antibody specific for the virus. The virus-specific antibody will adhere only if the virus is present. Thus, this assay is both qualitative and quantitative.

(ii) Competitive inhibition assays: Antigen can be detected in complex mixtures such as tissue extracts by its ability to inhibit the binding of an antibody to its antigen in solid phase assays. Continuing with the example of detecting virus in plant or insect extracts, such an assay (Fig. 11) would be performed as follows. Plastic wells of microtiter plates are first coated with antigen (i.e., the virus). In this variation, tissue extracts suspected of containing virus are added along with labelled anti-virus antibody (either a monoclonal antibody or antiserum could be used). If the virus is present in the tissue extracts, it will compete with the virus adhering to the plastic wells for antibodies. Thus, the free virus will competitively inhibit the binding of the virus-specific antibody to the plate. The degree of inhibition provides a quantitative determination of virus present in the tissue extract and can be compared to non-inhibited control wells.

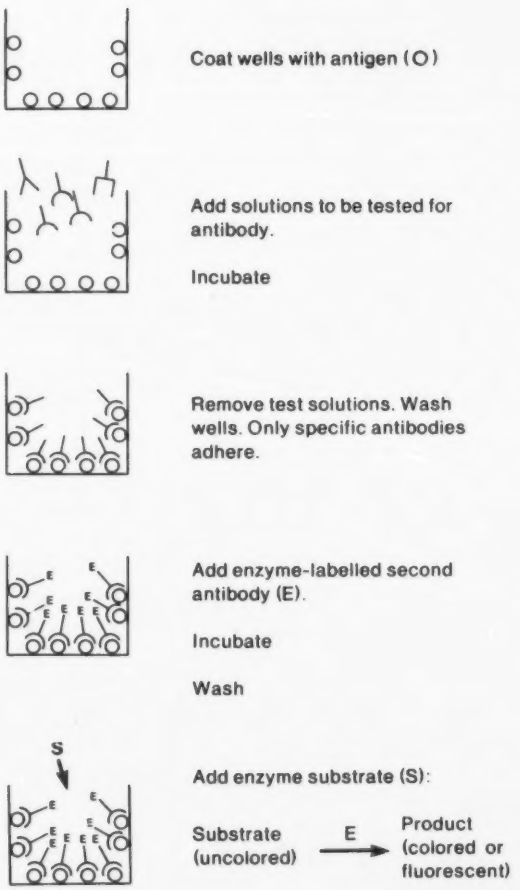
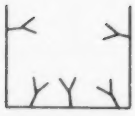


Fig. 9. Enzyme-linked immunosorbent assays (ELISA). These solid phase assays operate on a similar principle to the radioimmunometric assay (Fig. 8). However, in ELISA the second antibody is tagged with an enzyme instead of a radioisotope. Bound second antibody is determined by measuring a coloured reaction product when enzyme substrate is added to the wells.

These assays are extremely sensitive and rapid. Picograms (10^{-12} g) of antigen may be detected and results may be obtained in three to four hours.

RIA and ELISA are now being used to detect viral and other pathogens in both plant and insect tissues. For example, ELISAs using antisera have been used to detect prune dwarf, apple mosaic, potato leaf roll and tobacco ring spot viruses in leaf and other tissue extracts as well as, potato leaf roll virus and barley yellow dwarf viruses in their aphid vectors (*Myzus persicae* and *Sitobion avenae*) (Torrance and Jones 1982; Carlebach *et al.* 1982). Antisera have also been used in the serological diagnosis of *Prunus* necrotis ringspot virus in cherry orchards (Mink 1984). Mycologists have used ELISAs with antisera to



Coat well with virus-specific monoclonal antibody (Y).

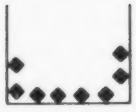
Add tissue extract suspected of containing virus (♦).

Incubate. Wash.

If virus present it will remain bound to the antibody in the well.

Add radioiodinated (*) anti-virus monoclonal antibody or antiserum. If virus is present, labelled antibody will bind and will remain after the wells are washed.

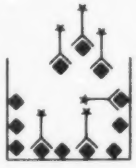
Fig. 10. Sandwich assays. These assays may be used to detect antigen such as a virus in a complex mixture such as a plant tissue extract. Microtiter plate wells are coated with virus-specific antibody (instead of antigen as in standard assays) then overlaid with the tissue extract. Virus present in the tissue extract will be pulled out and will remain firmly attached to the antibody-coated well. The presence of virus in the wells may be confirmed by adding more virus-specific antibody and detecting this as in routine radioimmunometric assays or ELISAs.



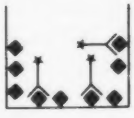
Coat microtiter plate wells with antigen e.g. virus (♦).

Add labelled anti-virus antibody (*) and tissue extract to be tested for virus to antigen-coated wells.

Allow competition for antibody to occur.



Antibody will bind to both free virus (in the tissue extract) and adherent virus (plastic wells). Wash.



Determine the amount of bound antibody.

Fig. 11. Competitive inhibition assays. An antigen such as virus in a complex mixture such as plant tissue extract may be quantitated by its ability to inhibit the binding of virus-specific antibody to virus-coated microtiter plate wells. Virus present in the tissue extract will reduce the amount of antibody available to bind to the solid phase antigen in the well. Antibody bound to virus-coated wells is detected as in routine radioimmunometric assays or ELISAs.

detect fungal structural elements and toxic products such as: *Epichloë typhina* in tall fescue (Johnson *et al.* 1983; Johnson *et al.* 1982), phytotoxins of *Ceratocystis ulmi* in elm (Scheffer and Elgersma 1981), and *Phonia tracheiphila*, the causative agent of Malsecco disease in lemon and other citrus trees (Nachmias *et al.* 1979). Monoclonal ELISAs are being developed to detect *Tristeza* virus in citrus trees (Dr. D.J. Gumpf, Dept. of Plant Pathology, University of California at Riverside, personal communication). Dorworth, *et al.* 1977; Dorworth 1974; Dorworth and Krywienczyk 1975) and others (Wendler *et al.* 1981) have used other less sensitive serologic techniques to study races of *Gremmeniella abietina* (the causative agent of scleroderris canker of conifers) differing in virulence.

Antisera or monoclonal antibodies have been employed in solid phase assays to distinguish between viral subtypes and different species of fungi. For example, ELISAs using antisera have distinguished between species of carlaviruses (Adams and Barbara 1982) and different species of endomycorrhizal fungi (Aldwell *et al.* 1983). Monoclonal antibodies have been used successfully in ELISA to distinguish plant viruses which are poorly immunogenic in rabbits and thus, cannot be studied with conventional antisera (Dr. E. L. Halk, American Type - Culture Collection, Rockville, MD., personal communication). Similarly, monoclonal antibodies directed to *Fusarium oxysporum* have been able to distinguish this fungal species from other *Fusarium* species (Ianelli *et al.* 1983). However, closely related species are not always distinguishable using immunologic techniques. Banowetz and his coworkers (Banowetz *et al.* 1984) were unable to detect immunologic differences (with antisera and monoclonals) between *Tilletia controversa* and *T. caries*, two closely related wheat fungi which are significant in wheat exportation.

Monoclonal antibodies and immunoassays have been used to study molecular changes during plant development and to determine biochemical phylogenetic relationships. For example, monoclonal antibodies have been used to compare and quantitate soluble and membrane forms of *Avena sativa* phytochrome (Partis *et al.* 1984) and to show differential expression of these two forms in seedlings grown at different light intensities. Yee and his associates (Yee *et al.* 1984) have used ELISA to compare bacterial and plant chloroplast thioredoxins, while others (Singh and Preiss 1984) have employed monoclonal antibodies and polyclonal antisera in ELISAs to study starch and glycogen branching enzymes in bacteria and maize.

Monoclonal antibodies are now being used in solid phase assays to measure levels of plant growth regulators and to evaluate host biochemical responses to disease. For example, monoclonal antibodies have been used in immunoassays to measure abscissic acid, indole-3-acetic acid and various other cytokinins in plant extracts (Mertens *et al.*1984). In forestry research, antibodies prepared to zeatin riboside and isopentenyladenosine extracted from Douglas-fir vegetative buds are being used in immunoassays to measure levels of these cytokinins as predictors of cone production (Morris *et al.* 1984). Hahn and coworkers (Hahn *et al.* 1984) have used radioimmunoassays to measure the phytoalexin, glyceollin I in soybean roots infected with the fungus *Phytophthora megasperma*.

Immunoassays such as those described above could be readily adapted for the detection of forest pathogens in seeds, seedlings or mature trees, as well as in insect vectors. Immunologic assays afford several advantages over currently used tests. For example, the identification of seed-borne pathogens using immunologic techniques would be advantageous for seed certification. Seed-borne pathogens are presently detected by germinating seed samples or testing seedlings (for instance, by inoculating tissue extracts into appropriate indicator hosts). These tests are both time consuming and expensive. In contrast, testing seed or seedlings for the pathogen using solid phase assays takes a matter of hours and is relatively inexpensive. Furthermore, current testing methods detect pathogens in embryos and seedlings only, whereas immunologic techniques would permit pathogen detection in ungerminated seed. A further advantage is that testing may be done at any time during the year.

Immunoassays may be used to monitor levels of insect-borne pathogens or to measure levels of various insect pathogens used in biologic control. The speed and sensitivity of these assays would facilitate the management of insect-borne disease. Their application in monitoring the levels of such biologic control agents as nucleopolyhedrosis virus in Douglas-fir tussock moth larvae

might aid in determining optimum times and amounts of virus application.

Immunofluorescence and immunohistochemistry

Antisera or monoclonal antibodies may be used as probes to detect any antigenic substance in plant or insect tissues using fluorescence microscopy. The specific antibody itself may be tagged with a fluorochrome such as fluorescein or rhodamine (direct immunofluorescence) or a fluorescent second antibody may be used (indirect immunofluorescence). Alternatively, the specific antibody may be tagged with an enzyme, such as peroxidase, which will convert a substrate to a colored reaction product. Antibodies tagged with electron-dense materials such as ferritin are useful for ultrastructural studies with electron microscopy.

Polyclonal antisera and monoclonal antibodies have been used in a variety of immunohistochemical applications in plant research. For example, immunofluorescence has been used to distinguish vesicular-arbuscular mycorrhizal (VAM) fungi from other fungal species (Wilson *et al.* 1983) and to differentiate several species of VAM fungi (Kough *et al.* 1983). Antisera have also been employed in immunofluorescence microscopy to localize *Ceratocystis ulmi* toxins in elm (Ipsen and Abul-Hajj 1982) and *Phaeolus schweinitzii* (conifer root and butt rot fungus) propagules in soil samples (Dewey *et al.* 1984). Immunohistochemistry has been used both to visualize fungal hyphae and to localize phytoalexins in host tissue in soybeans infected with *Phytophthora megasperma* (Hahn *et al.* 1984). Antisera and monoclonal antibodies have been employed in immunohistochemical studies of plant development. For example, intracellular transport of the lectin, phytohemagglutinin has been followed immunocytochemically in the axes and cotyledons of *Phaseolus vulgaris* seeds (Greenwood and Chrispeels 1984). Monoclonal antibodies have been used in immunofluorescence tests to study cell wall development in algae (Vreeland and Laetsch 1984) and phytochrome synthesis in *Avena* chloroplasts under differing light conditions (McCurdy and Pratt 1984). Also, antisera have been employed in immunohistochemical studies of legume protein processing (Herman and Shannon 1984)) and *Avena* globulin localiza-

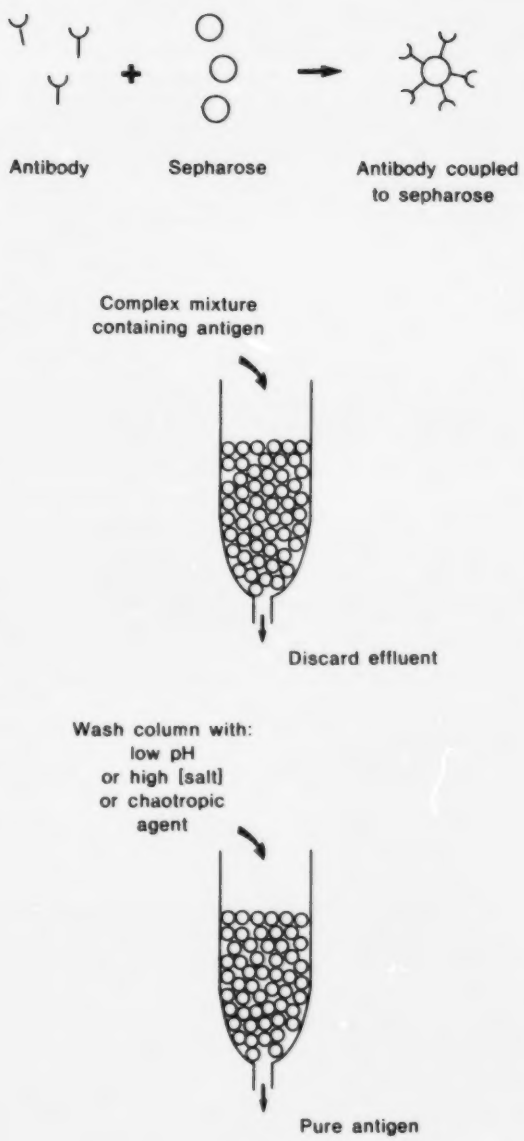


Fig. 12. Immunoaffinity chromatography. Antibodies may be used to selectively isolate their antigens from complex mixtures by affinity chromatography. Specific antibodies are coupled to a chromatographic medium such as agarose and poured into a column. When the complex mixture is passed through the column, antigen adheres to the Sepharose-bound antibodies while all other elements pass on through. Antigen can then be eluted from the column.

tion (Chan and Zee 1984) during several developmental stages.

Immunoaffinity chromatography

Monoclonal antibodies may be used in the isolation and purification of minor components in complex mixtures using affinity chromatography (Fig. 12). In this technique, antibody is chemically coupled to agarose beads (Sepharose 4B) which are then poured into a chromatographic column. The complex mixture containing the antigen to which the monoclonal antibody is directed is poured over the column. The antigen will bind to the antibody on the beads while the remainder of the mixture passes through the column and is discarded with the effluent. The antigen is then dissociated from the beads by washing the column with a low-pH or high-salt buffer or a chaotropic agent. Using affinity chromatography it is possible to achieve a 1000-fold enrichment. This method could be applied to the purification and enrichment of any substance to which an antibody has been prepared.

In both forestry and agricultural research this method has been used to purify growth regulators, enzymes or other interesting proteins for further study and characterization. For example, cytokinins from Douglas-fir vegetative buds have been purified by immunoaffinity chromatography (Morris *et al.* 1984) with specific antisera. Similarly, monoclonal antibodies are being employed in the purification of several other plant growth regulators (Mertens *et al.* 1984).

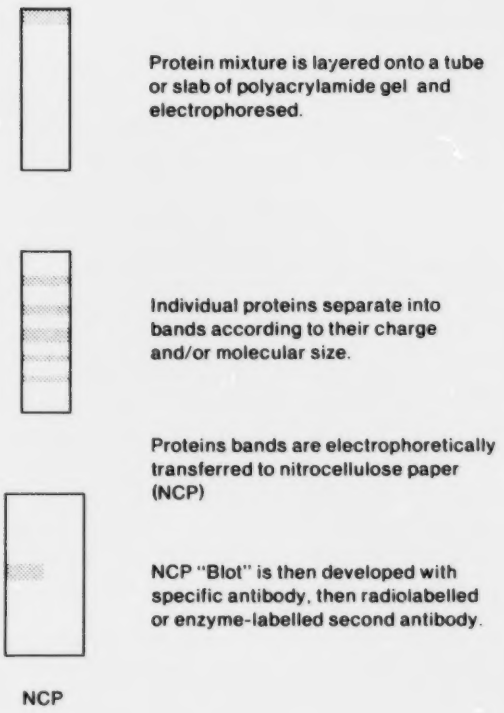

Protein mixture is layered onto a tube or slab of polyacrylamide gel and electrophoresed.
Individual proteins separate into bands according to their charge and/or molecular size.
Proteins bands are electrophoretically transferred to nitrocellulose paper (NCP)
NCP "Blot" is then developed with specific antibody, then radiolabelled or enzyme-labelled second antibody.
NCP

Fig. 13. Immunoblotting. Proteins (or other substances) separated by gel electrophoresis may be transferred electrophoretically to nitrocellulose paper (NCP). The NCP is then washed with antiserum. Specific antibodies will adhere to their respective protein bands (as originally obtained in gel electrophoresis). The NCP-bound antibodies may then be detected by enzyme-labelled or radioactively-labelled second antibodies.

Immunoblotting techniques

Antibodies may be used in conjunction with polyacrylamide gel electrophoresis in the study of proteins (and other biologic substances) using a technique called immunoblotting (Fig. 13) or "Western blotting" (Burnett 1981).

In the first step, a mixture of proteins such as a cell homogenate or tissue extract is applied to a slab of polyacrylamide gel. When an electrical current is passed through the gel each protein in the mixture will migrate according to its charge and/or molecular size and the mixture will be effectively resolved into separate protein bands. The polyacrylamide gel is then placed on a piece of nitrocellulose paper (NCP) and electrical current applied. The protein bands migrate onto the NCP in exactly the same relative positions they occupied within the gel- and stick. The "NCP blot" is then washed with antibody (usually an antiserum) which will bind to the band(s) representing the antigen. The bands containing bound antibody can then be detected by washing the NCP blot with radioactively-labelled second antibody and laying the washed and dried nitrocellulose paper against X-ray film. The radioactive bands show up as dark spots when the film is developed. Alternatively, enzyme-labelled

second antibody can be used and the bands detected colorimetrically (as in ELISA).

This technique has a great potential for the study of protein biochemical relationships and for the identification of specific proteins in complex mixtures. This technique has recently been applied to the study of genetic relationships among seeds from *Pinus strobus* (Eckert and Eckert 1984) and in comparative studies of *Avena* phytochrome (Partis *et al.* 1984) and plant acyl carrier proteins (Ohlrogge and Kuo 1984).

Immunoprecipitation

An alternate approach to locating antigens in complex mixtures is immunoprecipitation (Fig. 14). When specific antibody is mixed with a cell or tissue homogenate containing the antigen of interest the antibody will pull its antigen out of the mixture. In order to separate the antigen-antibody complexes *Staphylococci* are added to the mixture. These bacteria contain on their surfaces a substance called Protein A which binds to all rabbit antibody classes and some classes of mouse antibodies. The antigen-antibody complex sticks to the bacterial surface and can be centrifuged out of the mixture along with the bacteria. The antigenic substance binding to the antibodies can then be analyzed by polyacrylamide gel electrophoresis. The precipitated antigen can be seen by staining the gels or alternatively, radioactive precursors such as radioactively-labelled amino acids or sugars may be used to label cell constituents before immunoprecipitation. In the latter case the precipitated antigens can then be detected by autoradiography.

This technique was used to measure peroxidase release of peanut cells growing in culture (Van Huystee and Lobarzewski 1982). In this particular study biosynthesis and release of peroxidase were monitored by the incorporation of radioactively-labelled methionine and immunoprecipitation with anti-peroxidase antisera.

In forestry research similar techniques might be used to monitor the synthesis of growth regulators in vegetative buds under various environmental conditions or to measure the concentration of maturational hormones in insects at various developmental stages.

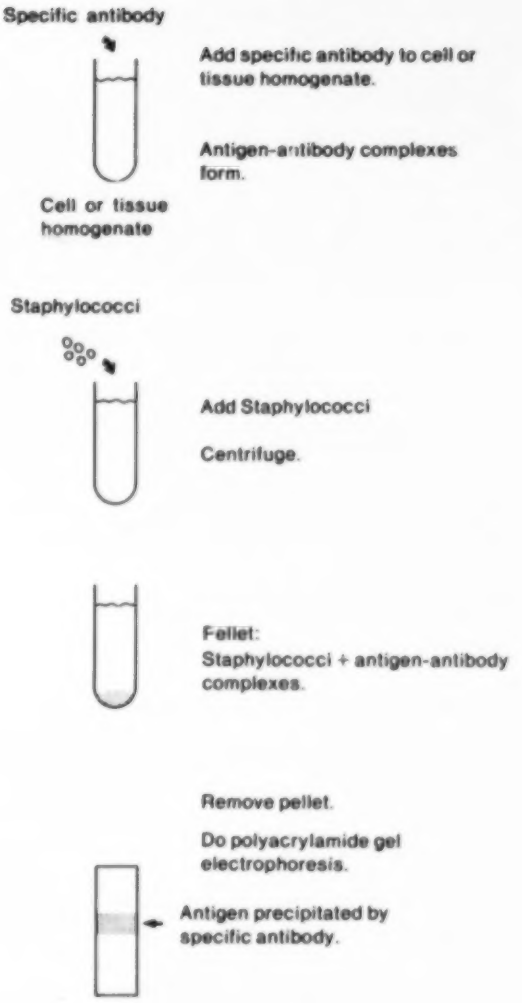


Fig. 14. Immunoprecipitation. Antisera or monoclonal antibodies may be used to pull their respective antigens out of complex mixtures by immunoprecipitation. Protein A-coated *Staphylococci* bacteria are added to facilitate precipitation of the antigen-antibody complexes. The antigenic components may then be visualized by doing gel electrophoresis of the precipitated material.

Two-dimensional polyacrylamide gel electrophoresis

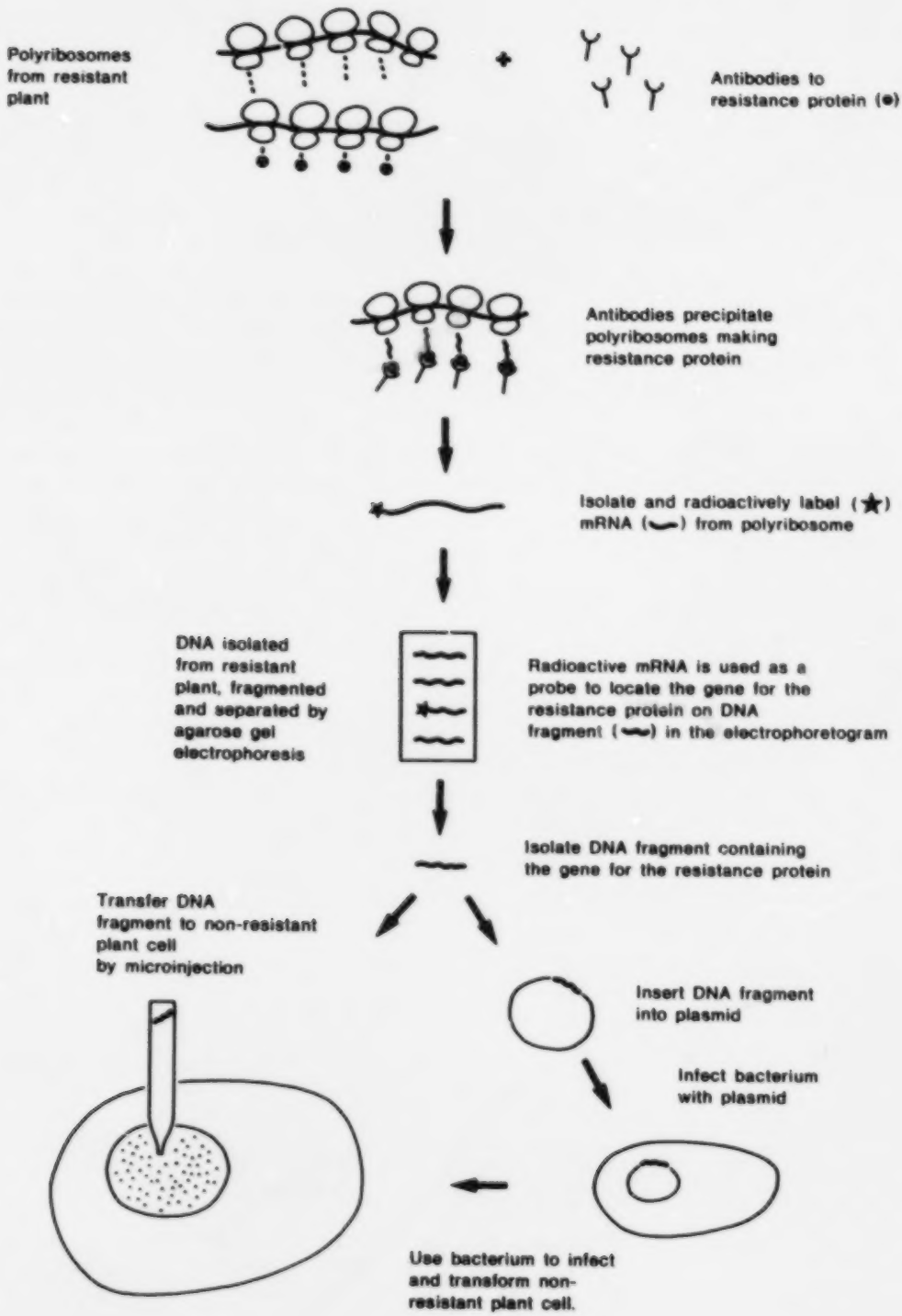
High-resolution two-dimensional polyacrylamide gel electrophoresis is a powerful technique for analyzing components of complex mixtures and can be used in conjunction with immunoprecipitation and Western blotting (Pearson and Anderson 1983; Pearson and Anderson 1982). It can also detect changes which occur in any tissue in response to disease (for instance, the increased synthesis of constituitive proteins or expression of new proteins, phytoalexins, etc.).

In the first dimension, the components of a complex mixture such as a cell or tissue homogenate or serum are separated according to charge by isoelectric focusing in tube gels. A pH gradient is generated in the gel and each component of the mixture migrates to its isoelectric point (the location in the gel that has a pH at which the component has a net charge of zero). The tube gel is then placed at the top of a slab gel and electrical current is reapplied. The components migrate in this second dimension according to their molecular size.

Figure 15 shows a two-dimensional gel of mouse serum proteins. When sensitive silver stains are used, nanogram (10^{-9} g) amounts of protein may be detected in the spots. Comparable or greater sensitivity may be obtained when biosynthetically radioactively-labelled mixtures are studied.

Two-dimensional gel electrophoresis has been used to study the effects of various hormonal treatments on protein synthesis in wing epidermal cells during early adult and second pupal stages of the silkmoth, *Antherea polyphemus*. In this case, the appearance of new proteins was monitored by biosynthetically labelling with radioactively-labelled methionine (Bryzyski and Ruh 1982).

Fig. 15. Two-dimensional polyacrylamide gel electrophoresis of mouse serum proteins. In the first dimension, serum proteins were separated according to their electrical charge by isoelectric focusing in polyacrylamide tube gels. The focused tube gel was then placed on top of a 10 to 20% polyacrylamide gradient slab gel and re-electrophoresed. In this second dimension, proteins migrated according to their molecular size and weight.


Polyribosomes from resistant plant
Antibodies to resistance protein (●)
Antibodies precipitate polyribosomes making resistance protein
Isolate and radioactively label (★) mRNA (⌣) from polyribosome
DNA isolated from resistant plant, fragmented and separated by agarose gel electrophoresis
Radioactive mRNA is used as a probe to locate the gene for the resistance protein on DNA fragment (⌣) in the electrophoretogram
Isolate DNA fragment containing the gene for the resistance protein
Transfer DNA fragment to non-resistant plant cell by microinjection
Insert DNA fragment into plasmid
Infect bacterium with plasmid
Use bacterium to infect and transform non-resistant plant cell.

Genetic engineering

New proteins or other biologic substances have been associated with superior genetic characteristics such as disease resistance. For example, new soluble proteins appear in tobacco plants when resistance is induced either by a hypersensitivity reaction to infection with TMV or by chemicals. Studies using classical genetic techniques indicate a single gene regulates the production of these resistance proteins (Ahl *et al.* 1982).

Once biologic substances such as resistance proteins in plants have been identified, specific antisera or monoclonal antibodies may be used to locate the genes for these proteins using a technique presently employed in genetic engineering in several other systems. In brief, specific antibody is used to precipitate the polyribosomes engaged in the synthesis of the protein of interest (Fig. 16). Once these polyribosomes have been separated from the rest of the cell's protein-synthesizing machinery, the messenger ribonucleic acid (mRNA) associated with the polyribosome may be isolated. This mRNA, a genetic "mirror image" of the deoxyribonucleic acid (DNA) coding for the protein, may be radioactively-labelled, then used as a probe to locate the gene on fragments of DNA isolated from the cell. Once the gene has been located on a DNA fragment, the fragment may be directly transferred to plant protoplasts by microinjection (Rasmussen *et al.* 1984). Alternatively, the DNA fragment carrying the gene may be inserted into plant cells in the following way. The isolated gene fragment is coupled with a plasmid, a small piece of extrachromosomal DNA which is able to infect bacterial cells such as *Agrobacter tumefaciens* (Rempel *et al.* 1984; Caplan *et al.* 1983). The bacteria containing the altered plasmid are then, in turn, used to transform (infect) plant cells. In this way a new gene may be integrated into the plant cell DNA. Thus, with genetic engineering techniques genes for disease resistance might be transferred to plant or tree cells growing in tissue culture. Presently, several laboratories are actively engaged in the construction of vectors for the transmission of new DNA into plant cells (Rempel *et al.* 1983).

A recent example of the contribution of genetic engineering to plant resistance is in the identification and localization of the structural gene for the insecticidal agent δ-endotoxin in *Bacillus thuringiensis* (Gonzalez *et al.* 1982). This gene is conveniently located on a plasmid in *B. thuringiensis*. Under certain conditions this plasmid will infect other bacteria. In this way the δ-endotoxin gene may be transferred to residual bacteria normally found on plants and trees. Of course, success is dependent upon the ability of these bacteria to express the gene and to secrete δ-endotoxin.

Fig. 16. Monoclonal antibodies and genetic engineering. Monoclonal antibodies recognizing a beneficial element, such as a resistance protein, may be used to selectvely immunoprecipitate polyribosomes engaged in its synthesis as they will react with the nascent protein chains on the ribosomes. Messenger RNA may then be removed from the polyribosomes, radioactively-labelled and used as a probe to locate the gene coding for the resistance protein on DNA fragments that have been separated by agarose gel electrophoresis. When the resistance gene has been localized on one of these fragments, that fragment may be excised from the agarose gel and transferred by microinjection to a nonresistant plant cell growing in tissue culture. Alternatively, the DNA fragment may be inserted into a plasmid which may then be used as a vector to transfer the gene, via a transforming bacterial species such as *Agrobacter tumefaciens*, into a nonresistant plant cell.

Conclusion

The fine specificities of antibodies — especially monoclonal antibodies — for their antigens make them excellent probes for detecting substances which may be present in miniscule amounts in complex biological systems.

This information report has described the principles and methodology involved in generating antisera and monoclonal antibodies. The theory behind several immunologic techniques employing these sensitive reagents has also been given. Current and potential applications of these rapid and economical techniques to research problems in forestry and agriculture have been described.

References

Adams A.N. and D.J. Barbara 1982. The use of $F(ab')_2$-based ELISA to detect serological relationships amongst carlaviruses. Ann. appl. Biol. 101: 495-500.

Ahl P., A. Cornu and S. Gianinazzi 1982. Soluble proteins as genetic markers in studies of resistance and phylogeny in *Nicotiana*. Phytopathology 72: 80-85.

Aldwell F.E.B., I.R. Hall and J.M.B. Smith 1983. Enzyme-linked immunosorbent assay (ELISA) to identify endomycorrhizal fungi. Soil Biol. Biochem. 15: 377-378.

Banowetz G.M., C.J. Trione and B.B. Krygier 1984. Immunological comparisons of teliospores of two wheat bunt fungi, *Tilletia* species, using monoclonal antibodies and antisera. Mycologia 76: 51-62.

Bryzyski R.G. and M.F. Ruh 1982. Protein synthesis during early adult and second pupal development in the silkmoth *Antherea polyphemus*. Insect Biochem. 12: 687-698.

Burnett W.N. 1981. "Western Blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated Protein A. Anal. Biochem. 112: 195-203.

Caplan A., L. Herrera-Estrella, D. Inzé, E. Van Haute, M. Van Montagu, J. Schell and P. Zambryski 1983. Introduction of genetic material into plant cells. Science 222: 815-821.

Carlebach R., B. Raccah and G. Loebenstein 1982. Detection of potato virus Y in the aphid *Myzus persicae* by enzyme-linked immunosorbent assay (ELISA). Ann. app. Biol. 101: 511-516.

Chan H. T. and S. Y. Zee 1984. Immunofluorescent localization and immunochemical studies of the globulin of oat (*Avena Sativa* L.) seeds. Plant Physiol. 75, suppl: 159 (Abstr.).

Dewey F.M., D.K. Barrett, I.R. Vose and C.J. Lamb 1984. Immunofluorescence microscopy for the detection and identification of propagules of *Phaeolus schweinitzii* in infested soil. Phytopathology 74: 291-296.

Dorworth C. E., J. Krywienczyk and D. D. Skilling 1977. New York isolates of *Gremmeniella abietina* (*Schleroderris lagerbergii*) identical in immunogenic reation to European isolates. Plant Dis. Rep. 61: 887-890.

Dorworth C. E. 1974. Comparison of soluble proteins of *Ascocalyx abietis* and *Gremmeniella abietina* by serology and electrophoresis Can. J. Bot. 52: 912-922.

Dorworth C. E. and J. Krywienczyk 1975. Comparisons among isolates of *Gremmeniella abietina* by means of growth rate, conidia measurement and immunogenic reaction. Can. J. Bot. 53: 2506-2525.

Eckert R.T. and B.S. Eckert 1984. A procedure for study of immunochemical properties of megagametophyte tissue of *Pinus strobus* L. Can. J. For. Res. 14: 142-145.

Greenwood J. S. and M. J. Chrispeels 1984. Immunocytochemical localization of phytohemagglutinin in the axes and cotyledons of *Phaseolus vulgaris* seeds. Plant Physiol. 75 suppl.: 46 (Abstr.).

Gonzalez J.M., B.J. Brown and B.C. Carlton 1982. Transfer of *Bacillus thuringiensis* plasmids coding for δ-endotoxin among strains of *B. thuringiensis* and *B. cereus*. Proc. Natl. Acad. Sci. U.S.A. 79: 6951-6955.

Hahn, M. G., A. Bonhoff and H. Grisebach 1984. Localization and quantitation of glyceollin I using a specific radioimmunoassay in soybean roots infected with *Pyhtophthora megasperma* F. sp. *glycinea*. Plant Physiol. 75 suppl.:166 (Abstr.).

Herman E. M. and L. M. Shannon 1984. Immunocytochemical and lectin cytochemical evidence for Golgi apparatus mediated deposition of legume body proteins. Plant Physiol. 75, suppl.: 145 (Abstr.).

Ianelli D., R. Capparelli, F. Marzioan, F. Scala and C. Noviollo 1983. Production of hybridomas secreting antibodies to the genus

Fusarium. Mycotaxon 18: 523-532.

Ipsen J.D. and Y.J. Abul-Hajj. 1982. Fluorescent antibody technique as a means of localizing *Ceratocystis ulmi* toxins in elm. Can. J. Bot. 60: 724-729.

Johnson M.C., R.L. Anderson, R.J. Kryscio and M.R. Siegel 1983. Sampling procedures for determining endophyte content in Tall Fescue seedlots by ELISA. Phytopathology 73: 1406.

Johnson M.C., T.P. Pirone, M.R. Siegel and D.R. Varney 1982. Detection of *Epichloë typhina* in Tall Fescue by means of enzyme-linked immunosorbent assay. Phytopathology 72: 647-650.

Köhler G. and C. Milstein 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497.

Köhler G., H. Hengartner and M.J. Shulman 1978. Immunoglobulin production by lymphocyte hybridomas. Eur. J. Immunol. 8: 82-88.

Kough, J., N. Malajczuk and R.G. Linderman 1983. Use of the indirect immunofluorescent technique to study the vesicular-arbuscular fungus *Glomus epigaeum* and other *Glomus* species. New Phytol 94: 57-62.

McCurdy D. and L. H. Pratt 1984. Immunocytochemical detection of phytochrome during greening in *Avena*. Plant Physiol. 75, suppl.: 89 (Abstr.)

Mertens R., J. Eberle and E. W. Weiler 1984. Production and use of monoclonal antibodies for endogenous plant growth regulators. Plant Physiol. 75 suppl. 137 (Abstr.).

Mink G.I. 1984. Use of enzyme-linked immunosorbent assay results in efforts to control orchard spread of Cherry Rugose Mosaic Disease in Washington. Plant Dis. 68: 207-210.

Morris J. W., R. C. Durley, D. P. Lavender and J. B. Zaerr 1984. Cytokinins in vegetative buds of Douglas-fir: Correlation with cone production. Plant Physiol. 75, suppl: 90 (Abstr.).

Nachmias, A., M. Bar-Joseph, Z. Solel and I. Barash 1979. Diagnosis of Mal Secco disease in Lemon by enzyme-linked immunosorbent assay. Phytopathology 69: 559-561.

Nisonoff A., Introduction to Molecular Immunology, p. 47, 1982, Sinauer and Associates, Inc., Sunderland, Massachusetts.

Ohlrogge J. B. and T. M. Kuo 1984. "Western Blot" Analysis of plant acyl carrier proteins. Plant Physiol. 75, suppl.: 100 (Abstr.).

Partis M. D., B. R. Jordan and B. Thomas 1984. Comparison of soluble and membrane phytochrome in *Avena*. Plant Physiol. 75(1) suppl: 74 (Abstr.)

Pearson T.W. and N.L. Anderson 1983. The use of high-resolution two-dimensional gel electrophoresis for analysis of monoclonal antibodies and their specific antigens. in Methods in Enzymology, Vol. 92, Immunochemical Techniques Part E. Monoclonal Antibodies and General Immunoassay Methods pp. 196-219. J.J. Langone and H. Van Vunakis, *eds.* Academic Press, New York.

Pearson T. and N.L. Anderson 1980. Analytical techniques for cell fractions. XXVIII. Dissection of complex antigenic mixtures using monoclonal antibodies and two-dimensional gel electrophoresis. Anal. Biochem. 101: 377-386.

Rasmussen R., B. Ward and V. Knauf 1984. Microinjection of immobilized protoplasts. Plant Physiol. 75, suppl.: 141 (Abstr.).

Rempel H., R. C. Tait and C. I. Kado 1984. A novel foreign gene transfer system using a mini-Ti plasmid for genetic modification of plants. Plant Physiol. 75, suppl.:31 (Abstr.).

Scheffer R.J. and D.M. Elgersma 1981. Detection of a phytotoxic glycopeptide produced by *Ophiostoma ulmi* in elm by enzyme-linked immunospecific assay (ELISA). Physiol. Plant Pathol. 18: 27-32.

Singh B. K. and J. Preiss 1984. Immunological characterization of starch branching enzymes in maize and comparison with enzyme from

Escherichia coli B. Plant Physiol. 75 suppl. 160 (Abstr.).

Torrance L. and R.A.C. Jones 1982. Increased sensitivity of detection of plant viruses obtained by using a fluorogenic substrate in enzyme-linked immunosorbent assay. Ann. appl. Biol. 101: 501-509.

Van Huystee R.B. and J. Lobarzewski 1982. An immunological study of peroxidase release by cultured peanut cells. Plant Sci. Lett. 27: 59-67.

Vreeland V. and W. M. Laetsch 1984. Intracellular synthesis of *Fucus* cell wall antigens. Plant Physiol. 75, suppl.: 62 (Abstr.).

Wendler P. B., A. R. Gotlieb and D. R. Bergdahl 1981. A newly discovered serotype of *Gremmeniella abietina*. Can. J. Bot. 58: 1923-1928.

Wilson J.M., M.J. Trinick and C.A. Parker 1983. The identification of vesicular-arbuscular mycorrhizal fungi using immunofluorescence. Soil Biol. Biochem. 15: 439-445.

Yee B. C., N. A. Crawford and B. B. Buchanan 1984. Immunological and affinity studies on C_3 and C_4 thioredoxins. Plant Physiol. 75 (1) suppl: 152 (Abstr.).